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Combined bioremediation and enzyme production by *Aspergillus* sp. in olive mill and winery wastewaters

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ABSTRACT

Olive mill wastewaters (OMW) and vinasses (VS) are effluents produced respectively by olive mills and wineries, both sectors are of great economic importance in Mediterranean countries. These effluents cause a large environmental impact, when not properly processed, due to their high concentration of phenolic compounds, COD and colour. OMW may be treated by biological processes but, in this case, a dilution is necessary, increasing water consumption. The approach here in proposed consists on the bioremediation of OMW and VS by filamentous fungi. In a screening stage, three fungi (*Aspergillus ibericus*, *Aspergillus uvarum*, *Aspergillus niger*) were selected to bioremediate undiluted OMW, two-fold diluted OMW supplemented with nutrients, and a mixture of OMW and VS in the proportion 1:1 (v/v). Higher reductions of phenolic compounds, colour and COD were achieved mixing both residues; with *A. uvarum* providing the best results. In addition, the production of enzymes was also evaluated during this bioremediation process, detecting in all cases lipolytic, proteolytic and tannase activities. *A. ibericus*, *A. uvarum* and *A. niger* achieved the highest value of lipase (1253.7 ± 161.2 U/L), protease (3700 ± 124.3 U/L) and tannase (284.4 ± 12.1 U/L) activities, respectively. Consequently, this process is an interesting alternative to traditional processes to manage these residues, providing simultaneously high economic products, which can be employed in the same industries.

1. Introduction

Liquid wastes from agro-industrial processes like wineries and olive oil industries are sources of local water pollution. Generally, these industries are nearby and generate their wastes at the same time of the year. For this reason, a common process to treat both wastes is of great interest. Vinasses (VS) and olive mill wastewaters (OMW) are the main liquid wastes generated in wineries and olive mills, respectively. These effluents are harmful to sewage treatment plants due to the large amounts of organic and suspended matter (Rytwo et al., 2013).

VS are liquid wastes generated by the distillation process of lees and low quality wines. Like other winery wastes, VS present acidic pH values, high biochemical oxygen demand (BOD) and chemical oxygen demand (COD). These wastes contain dead microorganisms and other compounds of wine (e.g. phenols, acids, carbohydrates, mineral salts) (Salgado et al., 2010). Commonly, VS are released to surface waters causing significant environmental problems (Beltrán et al., 1999). The amount of VS generated is 8–15 times greater than the ethanol production (Strong, 2009). In addition, the VS are produced in large quantities in a short season, making its treatment a harder challenge (Vlyssides et al., 2010). In the past few years, multiple applications have been investigated to manage their proper treatment. Among other applications, VS have been proposed as a nutritional supplement for microorganism growth (Salgado et al., 2009), for the production of protein-rich fungal biomass (Nitayavardhana and Khanal, 2010) or as a co-product in grape marc composting (Paradelo et al., 2010).

OMW are stable emulsions composed of water, olive pulp remains and residual oil (Lanciotti et al., 2005). Due to their high content on phenolic compounds (PC), COD and long-chain fatty acids, OMW are toxic to microorganisms and plants, and cannot be directly disposed into the environment (Paraskeva and Diamadopoulos, 2006; Sierra et al., 2007; Iamarino et al., 2009; Zirehpour et al., 2014). A common use of OMW is as soil

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amendment, but it can cause phytotoxic problems and change physicochemical characteristics of soil (Mekki et al., 2007). To meet the environmental standards, aerobic and anaerobic biological processes, including anaerobic co-digestion with other effluents and composting, are predominantly used to treat OMW (Paraskeva and Diamadopoulos, 2006). Bioremediation is environmentally friendly and cost competitive in comparison with chemical decomposition (España-Gamboa et al., 2011). This treatment can reduce COD and phenolic content, thus facilitating further management before its disposal (Ntougias et al., 2012). Nevertheless, phenols can act as inhibitors of many microorganisms, when present at low concentrations, and may reduce the performance of biological treatments. Therefore, in many cases, it is necessary to dilute OMW in order to reduce its toxicity to microorganisms, increasing water consumption (Öngen et al., 2007; Mann et al., 2010; Jarboui et al., 2013; Lakhtar et al., 2010).

The mixture of OMW with other agro-industrial effluents, which have lower pollutant load, may be a way to improve biological treatment performances. With this approach, initial phenolic content and COD can be reduced, avoiding the inhibition of microorganisms without using water. For example, cheese whey's addition to OMW in a proportion of 90/10 (v/v) was necessary to achieve the highest colour and phenolic reduction during the biological treatment of OMW with *Lactobacillus paracasei* (Aouidi et al., 2009).

Aerobic treatments for OMW detoxification can be performed by bacteria and fungi (Aouidi et al., 2009; Gonçalves et al., 2009; Mann et al., 2010). Three types of fungi are commonly used: white rot fungi, *Aspergillus* spp. and several different yeasts (McNamara et al., 2008). Among the *Aspergillus* genera, *Aspergillus niger* and *Aspergillus terreus* have been the most studied species to reduce PC, COD and colour of OMW (Garcia-Garcia et al., 2000; Garrido-Hoyos et al., 2002). *A. niger* belongs to the so called black aspergilli group, which include presently 19 accepted taxa (Samson et al., 2007). New species belonging to this group have been isolated from grape berries as *Aspergillus uvarum* and *Aspergillus ibericus* (Perrone et al., 2008; Serra et al., 2006). Production of enzymes in OMW and their detoxification by *A. ibericus* have already been tested (Abrunhosa et al., 2012), but *A. uvarum* has not been studied yet.

The aim of the present work was to select novel strains of *Aspergillus* spp., which could decolourise as well as improve the bioremediation of OMW, and study their suitability to treat OMW and VS mixtures while producing enzymes.

2. Materials and methods

2.1. Characterisation of effluents

The OMW were collected from olive mill (Region of Trás-os-Montes) and VS were collected from winery (Region of Minho) in 2012. Both effluents were separately homogenized and stored at −20 °C until use.

Total nitrogen (TN) was determined by the test kit Hach Lange LCK 338. For COD determination, the test kit Hach Lange LCK914 was used according to the manufacturer method. Total organic carbon (TOC) in liquid residues was quantified by the test kit Hach Lange LCK 387. Reducing sugars were determined by the dinitrosalicylic acid (DNS) method, and protein was analysed according to the Bradford method (Bradford, 1976). Total phenols were assessed by the Folin–Ciocalteau method (Commission Regulation (EEC) No. 2676/90), using caffeic acid as standard. Total solids were analysed by oven-dried to constant weight at 105 °C.

2.2. Screening of fungi for OMW bioremediation on agar plates

Table 1 shows the strains of *Aspergillus* assayed in this work, all of them supplied by MUM culture collection (University of Minho, Braga, Portugal). Fungi were cultivated to observe mycelium growth and decolourisation of OMW in agar media formulated with undiluted OMW, OMW diluted in the proportion 1:1 and 1:10 (v/v) with a nutrient supplemented medium (3 g/L $NaNO_3$, 1 g/L K_2HPO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L $CaCl_2 \cdot 2H_2O$, 1 mL/L metal solution (10 g/L $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$)) or diluted 1:1 and 1:3 (v/v) with VS. Fungi were pre-incubated on malt extract agar (MEA) (2% malt extract, 2% glucose, 0.1% peptone and 2% agar) plates at 25 °C and stored at 4 °C before inoculation on OMW agar plates. Inoculum was placed into the centre of the corresponding dishes, the growth and the halo of OMW decolourisation were followed during five days by measuring diameters of mycelium growth and decolourisation halos by visual inspection.

2.3. Bioremediation process by submerged fermentation

Submerged fermentations were performed in 250 mL Erlenmeyer flasks containing 100 mL of culture medium. The three media studied were 100% OMW without supplementation; OMW:nutrient supplementation medium (1:1, v/v) and OMW:VS (1:1, v/v). Flasks with media were sterilised during 15 min at 121 °C. Spores stored at 4 °C in slant tubes were suspended in a solution (0.01% peptone and 0.01% Tween 80) by strong agitation. Once spores were suspended, all media were inoculated with 2 mL of spores' suspension (10^7 spores/mL, counted with Neubauer chamber). The flasks were then incubated at 25 °C without agitation. Samples were collected during fermentation. All experiments were performed in duplicate.

2.4. Analytical methods

Decolourisation of media in submerged fermentations was determined by measuring absorbance at wavelengths of 395 and 525 nm. The biomass dry weight was determined at the end of fermentation after filtration using common laboratory filters (Whatman Nº 1) followed by drying at 100 °C. COD and total phenols were measured by the methods previously described. Statistical analyses were performed using Statistica 5.0 software.

Protease activity was quantified by a spectrophotometric method (Charney and Tomarelli, 1947), which is based on the reaction of 0.5 mL of SSF extracts with 0.5 mL of 0.5% (w/v) azocasein in acetate buffer 50 mM (pH 5) at 37 °C during 40 min. After incubation, 10% (w/v) trichloroacetic acid was added to precipitate residual protein not hydrolysed by the proteolytic enzymes. The sample was centrifuged (3000 g, 5 min) and a solution of potassium hydroxide 5 N was added to the supernatant, producing a pinky-orange colour. The intensity of this coloration was measured at a wavelength of 428 nm. The blank was performed under the same conditions, but adding trichloroacetic acid before incubation. One unit of proteolytic activity was defined as the amount of enzyme that produced an increase of 0.01 in absorbance relatively to the blank per minute, under the assay conditions.

Lipolytic activity was determined by spectrophotometric method, which was carried out using *p*-nitrophenyl-butyrate (*p*-NPB) in sodium acetate buffer 50 mM (pH 5.6) as substrate. The reaction mixture was incubated at 37 °C for 15 min and was stopped by adding acetone (Gomes et al., 2011). The absorbance was measured at a wavelength at 405 nm. One unit of activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute, under the assay conditions.

Tannase activity was analysed by spectrophotometric method

Table 1
Evaluation of media decolourisation on agar plates.

Strain	Code	OMW 10%	OMW 50%	OMW 100%	OMW:VS (1:1)	OMW:VS (3:1)
A. niger	MUM 03.58	++	++	++	+++	+++
A. niger	MUM 92.13	++	++	++	+++	+++
A. niger	01UAs83	−	+++	+++	+++	+++
A. niger	01UAs181	−	+++	+++	+++	+++
A. niger	01UAs107	++	+++	+++	+++	+++
A. foetidus	01UAs162	++	+++	++	+++	+++
A. ibericus	03UAs268	+++	+++	+++	+++	+++
A. ibericus	MUM 03.49	++	+++	+++	+++	+++
A. ibericus	03UAs91	+++	+++	+++	+++	+++
A. ibericus	03UAs113	−	+++	+++	+++	+++
A. uvarum	01UAs128	++	+++	++	+++	+++

Data collected after 5 days of growth, except the mixture of OMW:VS that corresponds to the 4th day of growth.
+ positive, ring <2 cm.
++ positive, ring >2 cm.
+++ positive, ring >4 cm.
− without ring.

based on the formation of a chromogen between gallic acid and rhodanine (Sharma et al., 1999). One unit of activity was defined as a micromole of gallic acid released per minute, under the assay conditions (30 °C, 10 min).

3. Results and discussion

3.1. Screening of fungi

Three types of microorganisms (white rot fungi, *Aspergillus* spp. and yeasts) have been mainly used in several studies to examine the ability of fungi to bioremediate OMW (McNamara et al., 2008), although the use of white rot fungi is more common than the use of *Aspergillus*. However, it was observed that some species of *Aspergillus* are also able to reduce the COD and PC of OMW (Aissam et al., 2007; Öngen et al., 2007). Aissam et al. (2007) evaluated the reduction of PC and COD with two yeasts (*Candida boidinii* and *Geotrichum candidum*) and two fungi (*Penicillium* sp. and *A. niger*). They observed that *A. niger* removed more COD and PC than the others microorganisms. In addition, *Aspergillus* spp. can biodegrade products of OMW in shorter cultivation periods than white rot fungi (Öngen et al., 2007).

Consequently, the growth of several *Aspergillus* strains belonging to the *A. niger* group was tested on agar plates to observe their ability to decolourise different concentrations of OMW and mixtures of OMW with VS. Table 1 shows that all strains were able to decolourise the plates with the different OMW media formulations in five days, with the exception of *A. niger* 01UAs83, *A. niger* 01UAs181 and *A. ibericus* 03UAs113 that did not grow on medium with 10% OMW, probably due to the low availability of carbon sources. The decolourisation of OMW may be due to the reduction of polyphenols which cause the dark colour of this waste. The OMW content on polyphenols depends on the age, type of olive and technology used (Yesilada et al., 1999). Undiluted OMW showed to be suitable for fungal growth; however, faster growth in media with diluted OMW was observed. The same conclusion was withdrawn in agar plates with mixtures of OMW and VS, where the maximum decolourisation and growth were achieved in four days. Öngen et al. (2007) reported the need to supplement OMW with nutrients, as yeast extract and minerals, to enhance microbial growth. Thus, the use of VS may replace the need to OMW supplementation with other nutrient sources, reducing the cost of process. Among all the fungi studied, *A. niger* MUM03.58, *A. uvarum* 01UAs128 and *A. ibericus* MUM 03.49 were selected to test OMW biodegradation in submerged fermentation.

3.2. Effect of VS addition on the biodegradation of OMW

To improve the process of bioremediation of OMW by fungi, different mixtures of OMW and VS were evaluated. Nitrogen, TOC, COD, reducing sugars, protein, lipids, total phenols and total solids were analysed in order to characterize these effluents and to compare them with data from the literature (Table 2). COD is an important parameter that measures the pollution load of effluents, being related with the organic load. In OMW, the organic load can be 100–150 times higher than in domestic wastewaters (Khatib et al., 2009). As shown in Table 2, in the effluents of this work, the COD in OMW was higher than in VS. However, observing not only those data reported in literature but also those determined in this work, it can be inferred that the limits can vary widely depending on the dilution effects caused by washing operations. On the contrary, the total nitrogen content in OMW was lower than in VS, since the latter contains dead cells of microorganisms, mainly yeasts and lactic acid bacteria, which are a source of nitrogen. For this reason, VS were already studied as a source of nutrients in different biotechnological processes (Salgado et al., 2009). The PC in OMW were higher than in VS. High levels of PC can inhibit microbial growth and can show phytotoxic effects. More than 30 different PC have been observed in OMW (McNamara et al., 2008). These levels of PC prevent the use of non-treated OMW for irrigation purposes in agricultural practices (McNamara et al., 2008).

Figs. 1–3 show the variations of pH, percentage of decolourisation and PC over time, during the biological treatment of undiluted and diluted OMW and the mixture OMW:VS (1:1) with the three selected fungi.

Fig. 1a–c displays the biodegradation of undiluted OMW, two-fold diluted OMW and OMW:VS (1:1, v/v) by *A. niger*. The pH dropped from 5.2 to 4.6 after twelve days of fermentation in undiluted OMW, this behaviour was similar in the mixtures of OMW with VS; however, in two-fold diluted OMW medium the decrease was higher. A decrease of pH was also observed during co-fermentation of OMW with cheese whey (Aouidi et al., 2009).

Decolourisation of OMW were analysed by measuring the absorbance at 395 nm and 525 nm over time. Table 3 shows the absorbance before and after the biological treatment. *A. ibericus* showed a higher decolourisation of undiluted OMW than the other fungi, achieving a colour reduction of 42.8% (at 525 nm) and 33.8% (at 395 nm) after 12 days. *A. niger* reduced moderately the colour of undiluted OMW, 33% (at 525 nm) and 25% (at 395 nm). *A. uvarum* exhibited no decolourisation of undiluted OMW, although it has been observed fungal growth in this medium. In a similar work,

Table 2
Main characteristics of effluents from wineries and olive oil industries.

Reference	VS				OMW			
	*	a	b	c	*	d	e	f
Nitrogen (mg/L)	218.67 ± 35.27	1354	740	1341	5.2 ± 0.2	250	3.4	n.d.
TOC (g/L)	3.53 ± 0.02	7.36	10.86	n.d	21.4 ± 1.2	n.d	15.3	n.d.
COD (g/L)	48.07 ± 1.43	21.72	27.5	121	122.9 ± 0.42	20	97	43.35
Reducing sugars (g/L)	0.68 ± 0.01	n.d.	n.d.	n.d.	12.7 ± 1.3	7.51	11.5	n.d.
Protein (g/L)	0.31 ± 0.08	n.d.	2.75	n.d.	0.04 ± 0.01	n.d	n.d.	n.d.
Lipids (g/L)	–	n.d.	0.25	n.d.	4.1 ± 0.42	n.d.	3.5	n.d.
Total phenols (g/L)	0.54 ± 0.03	0.74	n.d.	n.d.	5.91 ± 0.09	1.48	2.7	2.84
Total solids (g/L)	22.24 ± 0.09	0.75	36.6	10.27	15.48 ± 0.54	19.7	58	55.59

OMW: olive mill wastewater; TOC: total organic carbon; COD: chemical oxygen demand.
* Characteristics of effluents in current work (Salgado et al., 2014).
a: Beltrán et al., 1999, b: Vlyssides et al., 2010; c: España-Gamboa et al., 2012; d: Aouidi et al., 2009; e: Abrunhosa et al., 2012; f: Garrido-Hoyos et al., 2002.

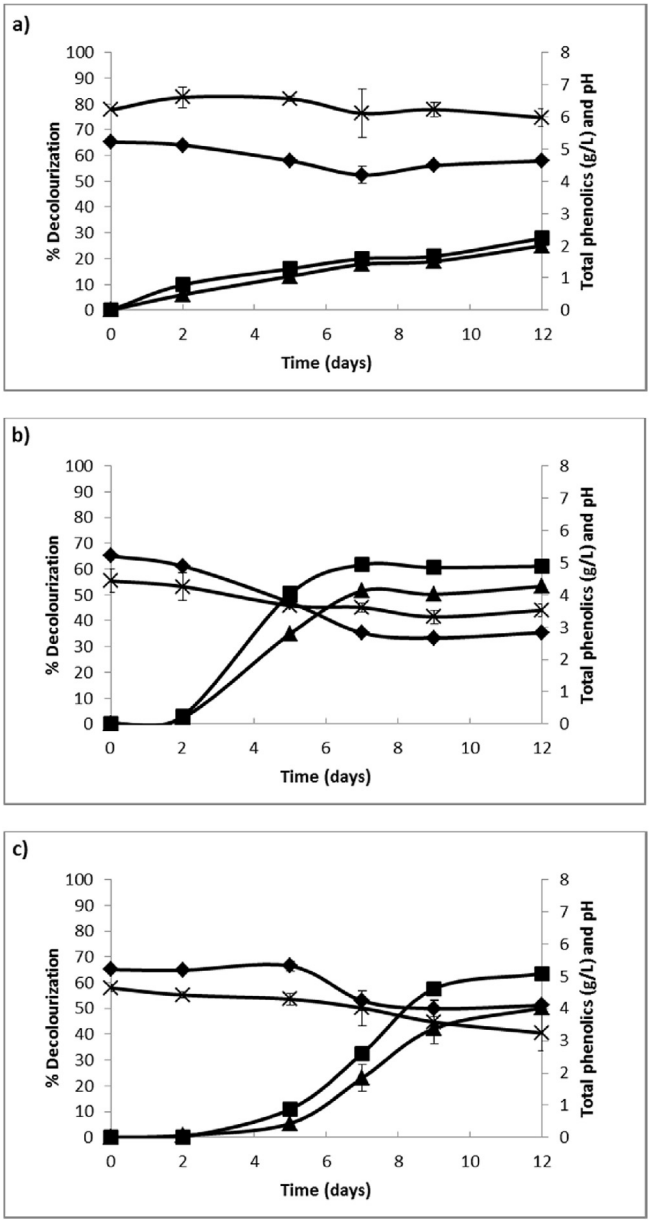

Fig. 1. Bioremediation of (a) undiluted OMW, (b) two-fold diluted OMW supplemented with nutrients, and (c) OMW:VS (1:1, v/v) with *A. niger* MUM 03.58. (×) PC, (◆) pH, and % decolourisation measured by reduction of absorbance at 395 nm (▲) and 525 nm (■).

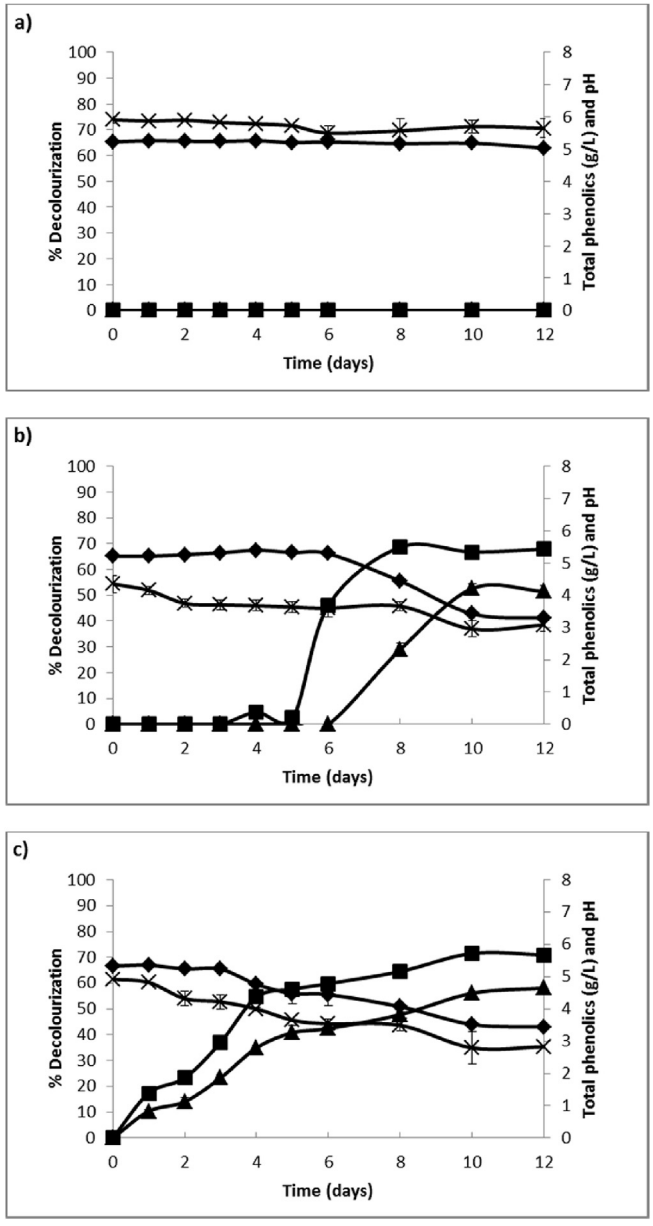

Fig. 2. Bioremediation of (a) undiluted OMW, (b) two-fold diluted OMW supplemented with nutrients, and (c) OMW:VS (1:1, v/v) with *A. uvarum 01UAs128*. (×) PC, (◆) pH, and % decolourisation measured by reduction of absorbance at 395 nm (▲) and 525 nm (■).

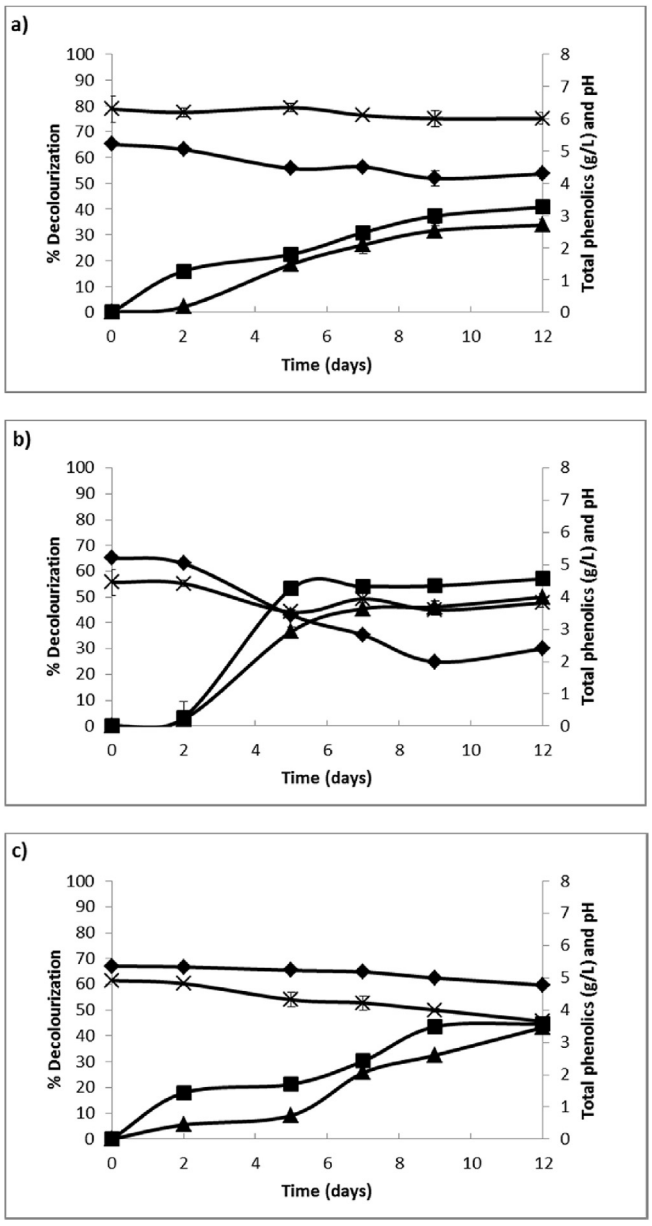

Fig. 3. Bioremediation of (a) undiluted OMW, (b) two-fold diluted OMW supplemented with nutrients, and (c) OMW:VS (1:1, v/v) with *A. ibericus MUM 03.49*. (×) PC, (◆) pH, and % decolourisation measured by reduction of absorbance at 395 nm (▲) and 525 nm (■).

Öngen et al. (2007) studied the effect of *A. niger* growth on OMW decolourisation, observing 43.8% colour reduction (at 390 nm) in two days, although using OMW with a lower colour intensity.

All strains improved the decolourisation of OMW after dilution and supplementation. *A. uvarum* provided the highest decolourisation in two-fold diluted OMW after 10 days: 68.9% at 525 nm and 52.6% at 395 nm. However, OMW dilutions involve more water consumption and nutrient supplementation, thus increasing the process costs. To avoid these problems, the dilution of OMW with VS is an interesting approach, since this effluent has a low pollutant load and can provide nutrients to improve fungal growth. Furthermore, both effluents are generated in the same geographical area, at the end of the year, making their co-treatment extremely convenient. All three strains achieved the highest decolourisation in OMW:VS (1:1, v/v). *A. uvarum*

Table 3
Biomass, COD, colour and total phenols before and after biological treatment of OMW.

Fungi	OMW (100%)						OMW (50%)						OMW:VS (1:1)					
	Biological treatment	Biomass (g/L)	COD (g/L)	C (Abs_{395})	C (Abs_{525})	TP (g/L)	Biomass (g/L)	COD (g/L)	C (Abs_{395})	C (Abs_{525})	TP (g/L)	Biomass (g/L)	COD (g/L)	C (Abs_{395})	C (Abs_{525})	TP (g/L)
A. ibericus	Before		129.9 ± 0.7	31.1 ± 0.4	13.9 ± 0.5	6.3 ± 0.1		79.2 ± 5.1	14.8 ± 0.6	6.8 ± 0.4	4.5 ± 0.4		92.6 ± 1.1	20.6 ± 0.1	8.85 ± 0.1	4.9 ± 0.1
	After	30.71 ± 0.33	115.8 ± 0.4	20.6 ± 0.9	7.9 ± 0.4	6.0 ± 0.2	18.59 ± 0.21	33.1 ± 2.7	7.4 ± 0.1	2.9 ± 0.1	3.8 ± 0.1	17.84 ± 0.15	60.8 ± 0.8	11.7 ± 0.2	4.9 ± 0.1	3.6 ± 0.2
	% red.		10.9 ± 0.5	33.8 ± 2.2	42.8 ± 0.5	4.6 ± 0.4		58.2 ± 2.7	50 ± 1.4	57.1 ± 1.8	14.2 ± 5.1		34.3 ± 0.8	43.2 ± 0.5	44.6 ± 0.6	26.5 ± 0.9
A. uvarum	Before		122.9 ± 0.4	27.4 ± 1.5	11.3 ± 0.4	5.9 ± 0.1		79.6 ± 1.4	15.9 ± 1.1	7.2 ± 0.9	4.4 ± 0.2		91.1 ± 7.7	20.6 ± 0.2	8.8 ± 0.4	4.9 ± 0.1
	After	24.60 ± 0.43	121.2 ± 0.3	34.9 ± 0.7	17.4 ± 1.1	5.7 ± 0.2	18.93 ± 0.34	35.2 ± 0.8	7.5 ± 0.3	2.2 ± 0.1	2.9 ± 0.2	27.43 ± 0.03	31.8 ± 2.9	9.1 ± 0.1	2.5 ± 0.1	2.8 ± 0.3
	% red.		1.4 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	3.6 ± 1.8		55.8 ± 0.8	52.6 ± 1.6	68.9 ± 3.6	32.2 ± 1.6		65.1 ± 3.1	56.1 ± 0.6	71.6 ± 1.5	43.3 ± 0.8
A. niger	Before		125.7 ± 4.4	33.6 ± 0.1	16.2 ± 0.5	6.2 ± 0.1		77.2 ± 4.8	14.8 ± 0.4	6.6 ± 0.3	4.4 ± 0.4		89.5 ± 5.5	19.1 ± 0.9	7.1 ± 0.6	4.6 ± 0.1
	After	28.95 ± 0.13	116.8 ± 3.2	25.2 ± 1.7	10.8 ± 1.1	5.9 ± 0.3	20.34 ± 0.54	39.8 ± 3.2	6.9 ± 0.1	2.6 ± 0.1	3.5 ± 0.2	28.97 ± 0.35	29.6 ± 2.1	9.5 ± 0.2	2.6 ± 0.1	3.2 ± 0.3
	% red.		7 ± 3.2	25 ± 5.1	33 ± 9.1	3.9 ± 5.4		48.4 ± 3.2	53.4 ± 2.1	61.1 ± 1.4	20.5 ± 2		66.9 ± 2	50.4 ± 1.3	63.7 ± 1.4	30.2 ± 2

OMW: olive mill wastewater; VS: vinasses; COD: chemical oxygen demand; C: colour; TP: total phenols; red.: reduction.

accounted for the highest decolourisation (71.6% at 525 nm and 56.1 at 395 nm) after 10 days of incubation.

Fungi show better decolourisation and biodegradation activities at acidic or neutral pH (Khan and Fulekar, 2013). The colour and phenols can be reduced by acidic treatments (Hamdi, 1993), thus a decrease of pH could cause a reduction of colour. To evaluate the effect of pH on the colour removal of OMW and OMW:VS (1:1, v/v), the absorbances at 395 and 525 nm were measured at different values of pH (Fig. 4a–b). In both cases, it was observed a reduction of colour about 25% at pH 4. Hamdi (1993) observed that acidic treatment of OMW only removed the polyphenols responsible for black colour.

Concerning phenols the lower removal was observed when undiluted OMW were assayed as basal media, in spite of the higher values achieved with *A. ibericus* (17.6% reduction). Nonetheless, the high concentration of initial phenols quantified in the OMW (>7 g/L) may have hindered the reduction of the PC. D'Annibale et al. (2004) observed a great improvement in biological reduction of phenolic compounds in OMW with a lower initial phenolic content. In two-fold diluted OMW, dephenolization improved with all fungi tested. *A. uvarum* obtained the maximum percentage of dephenolisation (32.2%) after 10 days. In the mixture OMW:VS (1:1, v/v), even higher reductions were observed. *A. uvarum* reached a dephenolisation of 43.3% after 10 days (Table 3). Thereby, diluting OMW with VS rather than with supplemented medium improved further the reduction of phenolic compounds. Öngen et al. (2007) achieved similar results in two-fold diluted OMW without any supplements. Using *Aspergillus tubingensis*, they achieved a 34.6% phenolic compounds removal; however, as stated before, the initial phenolic content of OMW was lower. In mixtures of OMW:cheese whey (1:1, v/v), biological reduction of phenolic compounds was 28.9% (Aouidi et al., 2009). Dephenolisation can also be achieved with adsorbent matrices as activated carbon, resins and zeolites. Some of them have even showed best results than fungal treatments when they were used at concentrations greater than 50 g/L (Padovani et al., 2013). However, the use of such matrices increases the cost of the process.

COD was measured before and after fungal treatment (Table 3). In undiluted OMW, the percentage of COD reduction was low. The best result was achieved with *A. ibericus* which removed 10.9% of initial COD. Two-fold diluted OMW, improved the removal of COD. *A. ibericus* and *A. uvarum* showed a similar percentage as COD reduction 58.2% and 55.8%, respectively. Once more, the mixture of OMW with VS improved the bioremediation process. *A. uvarum* and *A. niger* achieved reductions of 65.1% and 66.9% of the initial COD. In physicochemical treatment with granular activated carbon, reduction of COD of 69.7% was also achieved (Padovani et al., 2013). In mixture of OMW with cheese whey, *L. paracasei* reduced initial COD by 16% (Aouidi et al., 2009). Biological treatment by *Phanerochaete chrysosporium* allowed to reduce COD of OMW with an initial value of 80 g/L in about 75% (Sayadi and Ellouz, 1995). Aissam et al. (2007) also evaluated several fungi to bioremediate OMW and observed that *A. niger* reduced 80% of COD in OMW with an initial content of 82 g/L, after an adaptation phase.

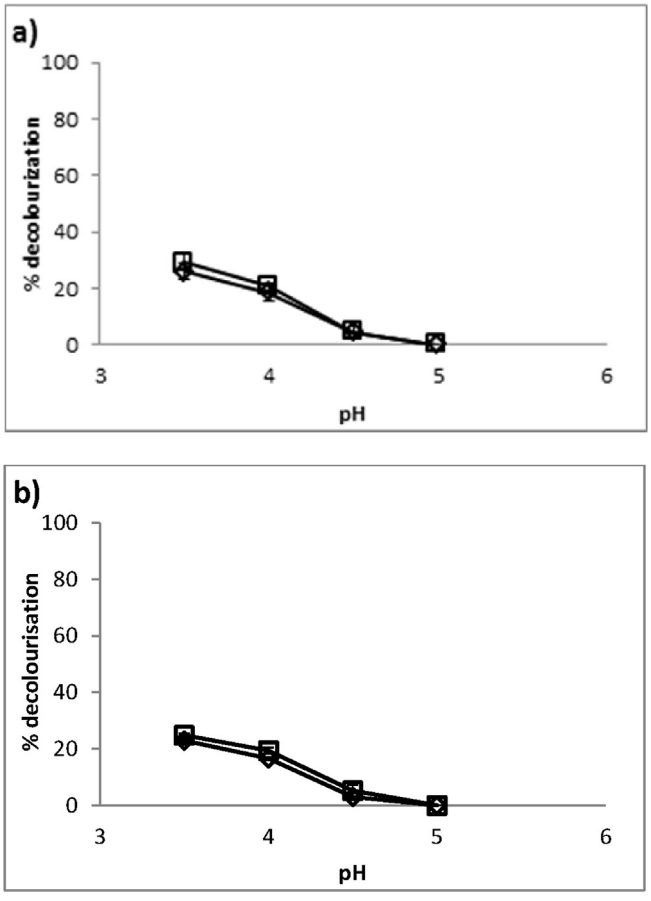

Fig. 4. Effect of pH in decolourisation of OMW (a) and OMW:VS (1:1, v/v) (b). Absorbance at 395 nm (□), absorbance at 525 nm (◊).

3.3. Enzymes production in the biodegradation processes

In addition to assessing the bioremediation of OMW and VS, enzymes production after fermentation with *Aspergillus* strains were determined. Proteolytic, lipolytic and tannase activities were identified in media after the bioremediation process. These enzymes can have application in laundry, since proteases and lipases are the key enzymatic constituents in detergent formulations to remove fatty and proteinaceous food stains (Grbavčić et al., 2011). Fig. 5a–c depict enzyme activities (U/L) in the three media fermented by *A. ibericus*, *A. uvarum* and *A. niger*. As it can be seen, the maximum proteolytic activity was obtained after bioremediation of OMW:VS by *A. uvarum* (3700 ± 124.3 U/L). The higher content of protein in VS may have improved proteases production by the three strains. *A. uvarum* showed specificity to produce proteases but low concentration of lipases and tannases were produced. *A. niger* is known to produce proteases; however, the production of proteases by *A. uvarum* and *A. ibericus* is not documented. In other works, with similar proteolytic activity analysis, protease production by *A. niger* in SSF was 5.27 U/mL (Couri et al., 2000), while *Bacillus subtilis* achieved 12 U/mL in submerged fermentation with synthetic medium (Soares et al., 2005). The maximum lipase activity (1253.7 U/L ± 161.2) was obtained by *A. ibericus* in fermentations with undiluted OMW. Except for *A. uvarum*, the highest lipase activity was observed in this medium, probably due to its higher lipids content, which act as inducer for the production of lipases. *A. ibericus* had already shown its ability to produce lipases, in OMW supplemented with mineral nutrients, a lipase activity of 2927.0 U/L was achieved in bioreactor batch cultures (Abrunhosa et al., 2012).

A low tannase activity was detected with all three strains; however, *A. niger* was clearly the best producer. Media with OMW and mixtures of OMW and VS were suitable for the production of tannases, *A. niger* produced 284.4 ± 12.1 and 137.1 ± 3.7 U/L, respectively. These media showed a higher content in PC which could have induced the tannase production (Aissam et al., 2005). Despite the higher content of PC of OMW (6.2 g/L) in this study, *A. niger* was not inhibited and produced tannase during the bioremediation process. Aissam et al. (2005) also studied tannase

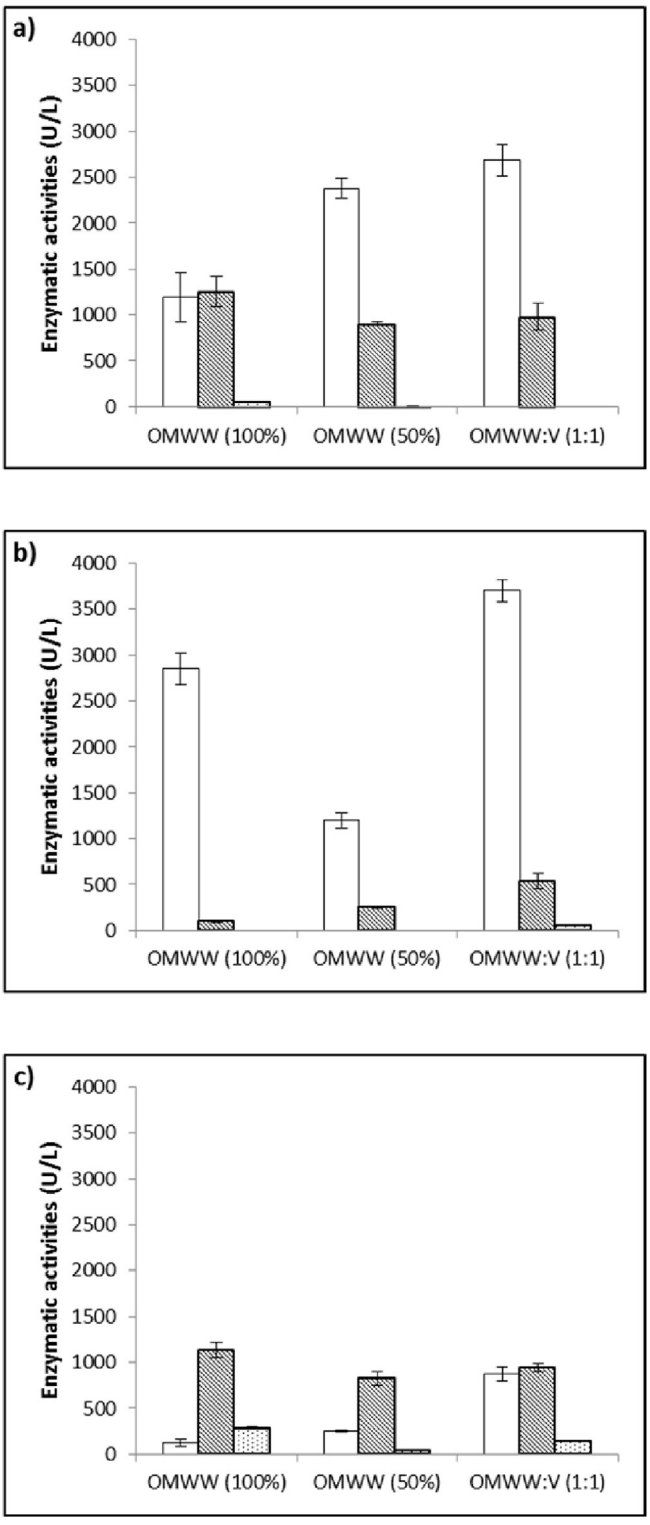

Fig. 5. Protease (clear), lipase (striped) and tannase (dotted) activities detected in effluents after the bioremediation with (a) *A. ibericus* MUM 03.49, (b) *A. uvarum* 01UAs128 and (c) *A. niger* MUM 03.58.

production by *A. niger* on four-fold diluted OMW and reported activities between 370 and 650 U/L. Results were slightly higher; however, in this study, OMW was not diluted and was not supplemented with nutrients.

4. Conclusions

Three *Aspergillus* strains, namely *A. niger* MUM03.58, *A. uvarum* 01UAs128 and *A. ibericus* MUM 03.49, were selected among different strains in a screening process conducted in Petri disks, to reduce PC, COD and colour of OMW. The best results were achieved in bioremediation processes using mixtures of OMW and VS, in the proportion 1:1 (v/v), particularly when using the strain *A. uvarum*. Consequently, a positive effect can be inferred from mixing OMW and VS to reduce toxic compounds of effluents.

Moreover, the bioremediation processes of these effluents allowed the simultaneous production of enzymes with industrial interest, as lipases, proteases and tannases. *A. ibericus*, *A. uvarum* and *A. niger* achieved the highest values of lipase, protease and tannase activities, respectively. A positive effect of mixture OMW and VS was also observed on proteases production.

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